

(19)



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European Patent Office
Office européen des brevets



(11) Publication number:

0 593 789 A1

(12)

EUROPEAN PATENT APPLICATION
published in accordance with Art.
158(3) EPC

(21) Application number: 93911978.0

(51) Int. Cl.⁵: **C12Q 1/68, C12Q 1/70,
C12N 15/00, C12N 15/51**

(22) Date of filing: 07.05.93

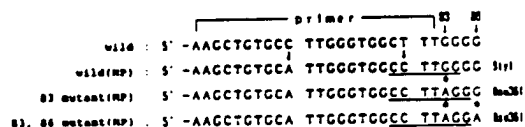
(66) International application number:
PCT/JP93/00602(67) International publication number:
WO 93/23567 (25.11.93 93/28)

(30) Priority: 08.05.92 JP 116293/92

(43) Date of publication of application:
27.04.94 Bulletin 94/17(64) Designated Contracting States:
DE GB IT(71) Applicant: **SUMITOMO METAL INDUSTRIES,
LTD.
5-33, Kitahama 4-chome
Chuo-ku
Osaka-Shi Osaka 541(JP)**(72) Inventor: **KOSHIZAKA, Takuya 701, 5-7-13
Sagamigaoka
Zama-shi
Kanagawa 228(JP)
Inventor: OKAMOTO, Hiroaki 3311-158
Oaza-Yakushiji
Minami-Kawachi-cho
Kawachi-gun Tochigi 329-04(JP)**(74) Representative: **TER MEER - MÜLLER -
STEINMEISTER & PARTNER
Mauerkircherstrasse 45
D-81679 München (DE)**(54) **METHOD OF JUDGING PRE-C MUTATION OF HEPATITIS B VIRUS.**

(57) A method of judging pre-C mutation of hepatitis B virus accurately in a short time by a simplified operation, which method comprises designing a primer set by introducing a mismatching base in the vicinity of a pre-C mutation site of a hepatitis B virus genome DNA in such a manner as to make it possible to amplify a given region containing the pre-C mutation site and to give a restriction enzyme cleavage pattern which varies according to whether or not the mutation has occurred in the amplified DNA, using the primer set to amplify the DNA by the polymerase chain reaction, and inspecting the pattern of cleavage by the restriction enzyme specific for the mutation, thus judging the pre-C mutation of hepatitis B virus or determining the percentage of the viruses thus mutated.

Fig. 1



Underline shows each restriction site of the
restriction enzyme on the right side.

Technical Field

The present invention relates to a process for detecting the presence or absence of one or more mutations within the precore (pre-C) region of hepatitis B viral genomic DNA, which is useful for predicting the prognosis of acute hepatitis B. The present invention also relates to a kit for use in the process.

Background Art

Hepatitis Be (HBe) antigen is observed in the circulatory system of individuals infected with hepatitis B viruses (HBV) in the initial phase of infection. However, during persistent infection, the HBe antigen gradually disappears and HBe antibody is then detected (seroconversion). It has been elucidated that this phenomenon results from the fact that during persistent infection, the wild type HBV which produces the initial infection gradually decreases in number and HBV mutants are produced which are incapable of secreting HBe antigen due to mutations within the pre-C region increasingly in persistent carriers. It is also known that HBV mutants incapable of secreting of HBe antigen have a stronger tendency to induce fulminant hepatitis B than does wild type HBV.

There is no known marker on the HBV viruses which is capable of predicting the prognosis of acute hepatitis B clinically. However, it is believed that predicting the prognosis of acute hepatitis B may be possible by analyzing the sequence of the pre-C region of the viral genome.

HBV mutants which are incapable of secreting HBe antigen have a point mutation from guanine (G) to adenine (A) at nucleotide 83 in the pre-C region. As a result, the codon encoding amino acid 28 is converted from TGG (Trp) to TAG (stop), which inhibits the synthesis of the precursor protein of HBe antigen and prevents secretion of HBe antigen. Furthermore, the mutants found in relatively severe cases of chronic hepatitis B accompanied by viremia often have point mutations from G to A at both nucleotides 83 and 86. In the past, detection of mutations in HBV has been carried out by amplifying the portion containing the mutation site of the DNA by means of the polymerase chain reaction (PCR) technique, linking this DNA fragment to an appropriate plasmid for cloning, isolating and purifying this recombinant, and sequencing. (See Y. Kosaka et al., Gastroenterology, Vol. 100, 1087-1094, 1991; H. Okamoto et al., J. Virology, Vol. 64, 1298-1303, 1990; H. Okamoto, Jikken Igaku, vol. 9, 1263-1268, 1991; Y. Akahane et al., Rinshouigaku, Vol. 16, 586-589, 1990)

However, this detection process takes much time and labor because of the required cloning and sequencing procedures and therefore is not suitable for treating many samples. Moreover, scores of clones have to be assayed simultaneously in order to determine the proportion of mutation when wild type HBV and mutants thereof coexist, and thus, the above process is not practical.

Accordingly, the object of the present invention is to solve the above-described problems of prior art methods, and to provide a process for detecting mutations in HBV and a kit for the detection of HBV mutations, which facilitate fast and precise detection of the mutations by simple procedures.

Disclosure of Invention

The present inventors have found that the detection of HBV pre-C mutants can be effected with greater ease and accuracy by using newly devised primers for amplifying the region which contains the mutation site within the pre-C region of the HBV genome by means of the mutation site within PCR technique.

The present invention provides a process for detecting one or more mutations within a pre-C region of hepatitis B viral genomic DNA in a sample which comprises hepatitis B viruses, comprising the steps of:

- (a) treating the sample to amplify a targeted region which contains a targeted mutation site within the pre-C region of hepatitis B viral genomic DNA by polymerase chain reaction using one or more primer sets, each of said primer sets being capable of amplification of the targeted region and including a primer which contains at least one intentionally introduced base mismatch corresponding to nucleotide(s) near the mutation site to be detected, said base mismatch resulting in the creation of a restriction site which enables amplified DNA products which contain the mutation to be distinguished from those which do not using a restriction enzyme which recognizes said restriction site;
- (b) treating the amplified DNA, which contains the base mismatch, with at least one restriction enzyme which recognizes the created restriction site contained in the amplified DNA;
- (c) fractionating the treated DNA or its fragments and identifying the treated DNA or its fragments; and
- (d) detecting the presence of hepatitis B viruses which contain the targeted pre-C mutation(s) and/or determining the proportion of the mutants which contain the mutation(s) by analyzing the digestion patterns obtained from the treatment with the restriction enzyme.

A primer set used in step (a) of the above process preferably comprises a first primer and a second primer. The first primer comprises a nucleotide sequence which corresponds to a DNA sequence near the pre-C mutation site, but which does not include the mutation site, and which primer contains at least one intentionally introduced base mismatch which results in the creation of a restriction site and thereby enables the mutation to be detected by treating the amplified DNA with one or more restriction enzymes which recognize the created site. The second primer comprises a nucleotide sequence which, together with the first primer, is capable of providing for amplification of the targeted DNA region which contains the mutation site or site(s).

One or more mutations in the HBV pre-C region can be detected according to the present invention, e.g., the mutation at nucleotides 83 and/or 86.

The present invention also relates to primer sets which are to be used in the above process, and further to kits suitable for detecting the mutations of HBV comprising this primer set and at least one restriction enzyme.

According to the present invention, HBV mutants can be detected. Additionally, the relative proportion of the mutant of HBV viruses, that is the proportion of HBV viruses which contain mutation can be also performed readily and precisely in short time.

Brief Description of Drawings

Fig. 1 is a schematic representation of a process for detection of the pre-C 83 mutation of HBV;
Fig. 2 is a schematic representation of a process for detection of the pre-C 86 mutation of HBV;

Best mode for Carrying Out the Invention

The procedure for detection according to the present invention includes the following steps:

- (1) amplifying a selected region of the HBV genome which contains one or more mutation site(s) by means of the polymerase chain reaction (PCR) technique using a primer set which is designed for detecting the mutations based on the difference in patterns of digestion of the amplified product with a restriction enzyme(s);
- (2) treating the amplified DNA with a restriction enzyme(s) and fractionating and identifying the thus treated DNA or its fragments by appropriate means such as electrophoresis;
- (3) analyzing the digestion patterns obtained by digestion with a restriction enzyme(s); and
- (4) detecting the mutations based on the restriction patterns or determining the ratio of wild type HBV to HBV mutants.

The first step of this process comprises the amplification of the targeted region of the HBV genome which contains the mutations. Samples suitable for use in detecting such mutations include samples containing HBV prepared from hepatitis B patient's blood, serum, plasma, lymphocyte, tissues and the like. The amplification procedure may be carried out by the commonly used PCR technique, or a two stage PCR technique (nested PCR technique) can alternatively be employed. The two stage PCR technique is one in which in the first stage, the amplification is carried out using a primer set capable of amplification of a longer portion than a target region, and in the second stage the amplification is carried out using a primer set capable of amplification of the target region within the portion amplified in the first stage.

The present process uses a primer sequence which contains at least one base mismatch in the vicinity of the mutation site which results in the creation of a restriction site specific to a selected mutation in the amplified DNA obtained using the mismatch primer. In the case of the two stage PCR technique, the primer which contains at least one base mismatch is utilized in the second stage.

In nature, no restriction site (restriction enzyme recognition site) is created which can distinguish the mutant from the wild type strain, when the G to A mutation at nucleotide 83 or 86 in the HBV pre-C region occurs. However, it is possible to create such a restriction site in this region by designing a primer containing at least one base mismatch. This is because a mismatch primer which only involves a few base mismatches, such as 1-5 mismatches, does not inhibit the amplification of the DNA when it is used in a PCR technique.

For example, as shown in Fig.1, a mismatch primer (MP) (on a sense chain) having a T to C change at nucleotide, hereinafter nt, 80 is used for the amplification to detect the mutation at nt 83. After amplification using this primer as a first primer, the amplified product has a new restriction site in the region including the mutation site. As a result, a *StyI* restriction site (CCWWGG) is created in the case of wild strains, and a *Bsu36I* restriction site (CCTNAGG) is created in the case of 83 mutants. Thus, by amplifying the DNA sequence by PCR using the primer having 22 nucleotides upstream from nt 82 which contains a mismatch

nucleotide at nt 80 as the first primer of the primer set, and then digesting the amplified product respectively with Styl or Bsu36I, or with both Styl and Bsu36I and identifying the resulting fragments by means of electrophoresis, wild strains and 83 mutants can be distinguished. Preferably, the primer is designed to further contain a C to A change at nt 70 so that the Styl restriction site which is created within the sequence between the first primer and the second primer may not be digested.

For detection of the mutation at nt 86, a mismatch primer (on an antisense strand) containing an A to T change at nt 92 and a T to C change at nt 96 is used (the expression of nucleotide in Fig.2 is based on the sense strand), and thereby a new restriction site can be created in the wild strains and the mutants, respectively. Thus, a Bcgl restriction site (10/12(N)GCA(N)6TCG(N)12/10) occurs in the wild strains and a Tth111I restriction site (GACNNNGTC) occurs in the 86 mutants. Therefore, the wild strains and the 86 mutants can be distinguished by digesting the product amplified by the PCR procedure using the primer having 24 nucleotides downstream from nt 88 which contains mismatch nucleotides at nt 92 and 96 as the first primer, with Bcgl or Tth111I, respectively, or with both Bcgl and Tth111I, and analyzing digested fragments by electrophoresis.

The second primer used along with the above first primer bearing the mismatch(s) in PCR procedure is one capable of producing along with the first primer amplified DNA which is detectable under appropriate conditions such as electrophoresis. This primer will only typically contain the introduced restriction site which enables detection of the mutation(s). However, other restriction sites may be present if they do not inhibit the detection of mutation(s). The desired amplified DNA can be obtained by the PCR technique using the primer set comprising the first and the second primer. Specifically, in Example 1 a first primer containing a mismatch for detection of 83 mutants and a second primer (on the antisense) containing the sequence downstream from the mutation site of HBV genome are used in the PCR procedure to produce a DNA 99 base pairs long, and the detection of 83 mutants in pre-C region is carried out by restriction enzyme treatment.

When the primer containing the T to C change at nt 80 is used for detection of 83 mutation, a DdeI restriction site (CTNAG) is also created in the case of 83 mutants. Thus, any other restriction enzyme suitable for detection of the mutation may be used. In designing the primer, the DNA sequence of the primer having a mismatch(es) for detection of mutation may be on a sense strand or an antisense strand of the DNA. Moreover, the object of the present invention can be also attained by designing a mismatch primer so that it can produce an amplified DNA having a single restriction site either in wild strains or in mutants, although in the above examples one restriction site is introduced in wild strains and another site is in the mutants. For example, when mismatch primer 5'-AAGCTGTGCCTTGGCCTGCTTT-3'(SIQ ID No.6) (G to C change at nt 75, T to C at nt 76, G to T at nt 77) is used for detection of 83 mutation, EcoNI restriction site (CCTNNNNNAGG) is created only in 83 mutants, and therefore DNA which can be digested with EcoNI is contained in the mutants.

Preferred primer sets used in the present invention are shown below. These primers may be synthesized by known methods, e.g., by using automatic DNA synthesizers.

Primer Set for Detection of pre-C 83 Mutation:

5'-AAGCTGTGCATTGGGTGGCCTT-3'(PC83F)(SEQ ID NO 1),
5'-AAAGAATTCAGAAGGCCAAAAAGA-3'(PC2)(SEQ ID NO 2);
5'-AAGCTGTGCATTGGGTGGCCTT-3'(PC83F)(SEQ ID NO 1),
5'-GGAGACTCTAAGGCCTCCCGA-3'(PC83R)(SEQ ID NO 3)

Primer Set for Detection of pre-C 86 Mutation:

5'-TTCTTTATACGGGTCGATGACCAT-3'(PC86R)(SEQ ID NO 4),
5'-GGAGGCTGTAGGCATAAATTGGT-3'(PC86F2)(SEQ ID NO 5).

The amplification by means of the PCR technique may be carried out conventionally by denaturing DNA, annealing the above primers to this, adding heat-stable polymerase and dNTP (dATP, dGTP, dCTP, dTTP) and extending the DNA chain. This repeated cycle of denaturing, primer annealing and DNA chain extension results in amplification of the desired target region.

After amplifying DNA by the PCR technique, the resulting product is treated with one or more restriction enzymes. The selected restriction enzyme will produce different digestion patterns depending on whether the mutation in the pre-C region is present or not. For example, in Example 1, Styl is employed as the restriction enzyme which enzyme is capable of digesting the amplified wild strain DNA product but is incapable of digesting the 83 mutant DNA, and Bsu36I is employed as the restriction enzyme which is

5

4

1

was carried out by 30 repeated cycles of denaturing (at 94°C for 30 seconds), annealing (at 55°C, 1 minute), DNA extension (at 72°C, 1 minute), and further DNA extension (at 72°C, 10 minutes).

About 200ng(10 µl) of the resulting amplified DNA were treated with a restriction enzyme, Styl or Bsu36I. According to a commonly used procedure, the resulting product was subjected to electrophoresis using 10% polyacrylamide gel, stained with ethidium bromide and exposed to UV light, resulting in the display of bands. Evaluation was made using the data shown in Table 1 which describes the fragment size of the band to be detected. When the product was digested only with Styl, there was no mutation in the pre-C region of HBV existing in the serum, and therefore all these viruses were of wild type. When the product was digested only with Bsu36I, all viruses existing in the serum were pre-C 83 mutants. If both coexisted, the intensity of the 99 bp band which resulted from no digestion with each restriction enzyme varied depending on the ratio of wild strains and mutants. The results are shown in Table 2.

Table 1

Data for determination		
	① Styl	② Bsu36I
83 wild (G)	80,19	99
83 mutant (A)	99	79,20

Table 2

Sample No.	Presence or absence of band (strong - weak + +- +)				Determination
	Styl		Bsu36I		
	99 bp	88 bp	99 bp	79 bp	
1	++			++	83 mutant
2	+			+	83 mutant
3		+	+		83 wild
4	++	+	+	++	83 wild, mutant
5		++	++		83 wild
6	+			+	83 mutant
7	+	+	+	+	83 wild, mutant

Example 2

Detection of 83 mutation in HBV pre-C region (2)

Pre-C 83 mutants were detected by the PCR technique as described below using the above-mentioned DNA sample solution. The primer designed for detection of the mutation in the nucleotide sequence on the viral genome was 5'-AAGCTGTGCATTGGGTGGCCTT-3'(PC83F)(SEQ ID NO 1), and the primer for producing 161 base pairs long DNA after amplification was 5'-GGAGACTCTAAGGCCTCCCGA-3'(PC83R)(SEQ ID NO 3). These primers, 20 µl of DNA sample solution, 2.5 units of heat-stable DNA polymerase, 10 mM Tris-HCl(pH 8.0), 50 mM KCl, 2.5 mM MgCl₂, 0.01% gelatin, 0.02% Nonidet P-40, dNTP(dATP, dCTP, dGTP, dTTP)(each final concentration: 200 µM) were mixed and the final volume was adjusted to 100 µl. Then, 50 µl of mineral oil were overlaid and the reaction was allowed to proceed. The amplification of DNA was carried out by 30 repeated cycles of denaturing (at 94°C for 30 seconds), annealing (at 55°C, 1 minute), DNA extension (at 72°C, 1 minute), and further DNA extension (at 72°C, 10 minutes).

About 200ng(10 µl) of the resulting amplified DNA were treated with a restriction enzyme, Styl or Bsu36I. According to a commonly used procedure, the resulting product was subjected to electrophoresis using 10% polyacrylamide gel, stained with ethidium bromide, and exposed to UV light, resulting in the display of bands. Analyzing the digestion pattern, evaluation was made using the data shown in Table 3.

When the product was digested only with Styl, there was not mutation in the pre-C region of HBV existing in the serum, and therefore, all these viruses were of wild type. When the product was digested only with Bsu36I, all viruses existing in the serum were pre-C 83 mutants. If both coexisted, the intensity of the 161 bp band which resulted from no digestion with each restriction enzyme varied depending on the ratio of wild strains and mutants. The results are shown in Table 4.

Table 3

Data for determination		
	① Styl	② Bsu36I
83 wild (G)	142,19	161
83 mutant (A)	161	141,20

Table 4

Sample No.	Presence or absence of band (strong ~ weak ; + + - +)				Determination
	Styl		Bsu36I		
	161 bp	142 bp	161 bp	141 bp	
1	+			+	83 mutant
2	+			+	83 mutant
3		+	+		83 wild
4	+	+	+	+	83 wild, mutant
5		+	+		83 wild
6	+			+	83 mutant
7	+	+	+	+	83 wild, mutant

Example 3

Detection of 83 mutation in HBV pre-C region (3)

Pre-C 83 mutants was detected by the Nested-PCR technique as described below using the above-mentioned DNA sample solution. 5'-AATGTCAACGACCGACCTTG-3'(\$012) and 5'-GGAAAGAAG-TCAGAAGGCAA-3'(\$030) were used as the primers of the first stage. These primers, 5 μ l of DNA sample solution, 1.25 units of heat-stable DNA polymerase, 10 mM Tris-HCl(pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, and dNTP(dATP, dCTP, dGTP, dTTP)(each final concentration: 200 μ M) were mixed and the final volume was adjusted to 50 μ l. Then, 25 μ l of mineral oil were overlaid and the reaction was allowed to proceed. The amplification of DNA was carried out by 35 repeated cycles of denaturing (at 94°C, 1 minute), annealing (at 55°C, 1 minute), DNA extension (at 72°C, 1 minute and 30 seconds), and further DNA extension (at 72°C, 7 minutes).

The primer designed for detection of the mutation in nucleotide sequence on the viral genome was 5'-AAGCTGTGCATTGGGTGGCCTT-3'(PC83F), and the primer for producing 99 base pairs long DNA after amplification was 5'-AAAGAATTCAGAAGGCAAAAAGA-3'(PC2). These primers, 1 μ l of the resulting PCR solution of the first stage, 1.25 units of heat-stable DNA polymerase, 10 mM Tris-HCl(pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, and dNTP(dATP, dCTP, dGTP, dTTP)(each final concentration: 200 μ M) were mixed and the final volume was adjusted to 50 μ l. Then, 25 μ l of mineral oil were overlaid and the PCR of the second stage was allowed to proceed. The amplification of DNA was carried out by 25 repeated cycles of denaturing (at 94°C for 1 minute), annealing (at 55°C, 1 minute), DNA extension (at 72°C, 1 minute and 30 seconds), and further DNA extension (at 72°C, 7 minutes).

About 200ng(10 μ l) of the resulting amplified DNA was treated with a restriction enzyme, Styl or Bsu36I. According to a commonly used procedure, the resulting product was subjected to electrophoresis

using 10% polyacrylamide gel, stained with ethidium bromide, and exposed to UV light, resulting in the display of bands. Analyzing the digestion patterns, evaluation was made using the data in Table 1. When the product was digested only with Styl, there was no mutation in the pre-C region of HBV existing in the serum, and therefore all these viruses were of wild type. When the product was digested only with Bsu36I, all viruses existing in the serum were pre-C 83 mutants. When both of these viruses coexist, the intensity of the 99 bp band which results after treatment with each restriction enzyme varies dependent on the ratio of the wild strains to the mutants. The same results as shown in Table 2 were obtained.

Example 4

Detection of 86 mutation in HBV pre-C region

Pre-C 86 mutants were detected by the PCR technique as described below using the above-mentioned DNA sample solution. The primer designed for detection of the mutation in nucleotide sequence on the viral genome was 5'-TTCTTTATACGGGTCGATGACCAT-3'(PC86R)(SEQ ID NO 4), and the primer for producing 149 base pairs long DNA after amplification was 5'-GGAGGCTGTAGGCATAAATTGGT-3'(PC86F2)(SEQ ID NO 5). These primers, 20 μ l of DNA sample solution, 2.5 units of heat-stable DNA polymerase, 10 mM Tris-HCl(pH 8.0), 50 mM KCl, 2.5 mM MgCl₂, 0.01% gelatin, 0.02% Nonidet P-40, and dNTP(dATP, dCTP, dGTP, dTTP)(each final concentration: 200 μ M) were mixed and the final volume was adjusted to 100 μ l. Then, 50 μ l of mineral oil were overlaid and the reaction was allowed to proceed. Amplification of DNA was carried out by 30 repeated cycles of denaturing (at 94 °C for 30 seconds), annealing (at 55 °C, 1 minute), DNA extension (at 72 °C, 1 minute), and further DNA extension (at 72 °C, 10 minutes).

About 200ng(10 μ l) of the resulting amplified DNA were treated with a restriction enzyme, Bcgl or Tth111I. According to a commonly used procedure, the resulting product was subjected to electrophoresis using 10% polyacrylamide gel, stained with ethidium bromide and exposed to UV light, resulting in the display of bands. Analyzing the digestion patterns, evaluation was made using the data shown in Table 5. When the product was digested only with Bcgl, there was no mutation in pre-C region of HBV existing in the serum, and therefore, all these viruses were of wild type. When the product was digested only with Tth111I, all viruses existing in the serum were pre-C 86 mutants. If both coexisted, the intensity of 146 bp band which resulted from no digestion with each restriction enzyme varied depending on the ratio of wild strains and mutants. The results are shown in Table 6.

Table 5

Data for determination		
	① Bcgl	② Tth111I
86 wild (G)	113,34,2	149
86 mutant (A)	149	126,23

Table 6

Sample No.	Presence or absence of band (strong ~ weak ; + + - +)				Determination
	Bcgl		Tth111I		
	149 bp	113 bp	149 bp	126 bp	
1	+			+	86 mutant
2		+	+		86 wild
3		++	++		86 wild
4	++	+	+	++	86 wild, mutant
5		++	++		86 wild
6		+	+		86 wild
7		+	+		86 wild

Industrial Applicability

According to the present invention, mutation(s) in the HBV pre-C region can be detected by analyzing the digestion pattern obtained from amplified DNA with particular primers. Therefore, the present invention provides a much improved method for detecting mutation(s) readily and accurately in a short time. Furthermore, the process is clinically applicable to samples in which wild strains and mutants coexist, and enables their relative ratios to be determined. Thus, the subject invention provides a convenient method for predicting the prognosis of acute hepatitis B infection based on the detection for the mutation(s) in the HBV pre-C region.

Sequence Listing

SEQUENCE IDENTIFIER NUMBER: 1

SEQUENCE LENGTH: 22

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid synthetic DNA

SEQUENCE

22

AAGCTGTGCA TTGGGTGGCC TT

SEQUENCE IDENTIFIER NUMBER: 2

SEQUENCE LENGTH: 24

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid synthetic DNA

SEQUENCE

24

AAAGAATTCA GAAGGCAAAA AAGA

SEQUENCE IDENTIFIER NUMBER: 3

SEQUENCE LENGTH: 21

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid synthetic DNA

SEQUENCE

21

GGAGACTCTA AGGCCTCCCG A

SEQUENCE IDENTIFIER NUMBER: 4

SEQUENCE LENGTH: 24

SEQUENCE TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: other nucleic acid synthetic DNA

SEQUENCE

24

TTCTTTATAC GGGTCGATGA CCAT

Underline shows each restriction site of the restriction enzyme on the right side.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP93/00602

A. CLASSIFICATION OF SUBJECT MATTER Int. Cl ⁵ C12Q1/68, 1/70, C12N15/00, 15/51 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) Int. Cl ⁵ C12Q1/68, 1/70, C12N15/00, 15/51 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) WPI, WPI/L, BIOSIS PRE VIEWS, GENETYX		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. Virology : 64 p. 1298-1303 (1990)	1-13
Y	Experimental medicine : 9 p. 1263-1268 (1991)	1-13
Y	Clinical medicine : 16 p. 586-589 (1990)	1-3
Y	JP, A, 2-31699 (Beilar Collage of Medisin), February 1, 1990 (01. 02. 90), & EP, A, 333465 & AU, B, 632996	1-3
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex		
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Date of the actual completion of the international search July 28, 1993 (28. 07. 93)		Date of mailing of the international search report August 17, 1993 (17. 08. 93)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.		Authorized officer Telephone No.